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**TITLE:** Evaluation of Androgen Receptor Function in Prostate Cancer Prognosis and Therapeutic Stratification

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14. ABSTRACT Although most prostate cancers are initially responsive to androgen ablation therapy, they become treatment resistant as tumor cells develop mechanisms to evade the treatment. Early knowledge of the androgen receptor dysfunctions will help in patient stratification for emerging therapeutic strategies. We proposed an approach for monitoring potential dysfunctions of the androgen receptor by measuring expression of a panel of genes directly regulated by androgen receptor. We examined human prostate cancer tissues (surgery or diagnostic biopsy specimens) at early stages of the disease and matched with longitudinal follow up data. Within the first reporting period we have completed the quality control of detecting PSA/KLK3, PMEPA1, NKX3.1, ODC1, AMD1 and TMPRSS2-ERG genes in VCap cell culture model monitoring kinetic and dose response to androgen. In the second reporting period we have completed the qRT-PCR evaluation of in 77 patients by monitoring ERG, PSA, PMEPA1 and GAPDH levels. Also, we have completed the evaluation of 40 whole mounted sections of RP specimens by immunohistochemistry assessing AR, ERG, NKX3.1 and PSA proteins and compared the results to corresponding GeneChip mRNA expression levels from the same tumor foci. The result indicated remarkable accuracy of the androgen regulated gene expression at mRNA levels performed better in prediction favorable outcomes in tumors with well differentiated morphology. By the completion of the proposed research we will provide a quantitative index of AR dysfunction for enhancing prognostic accuracy and to stratify patients for specific therapeutic approaches at early stages of prostate cancer treatment.				
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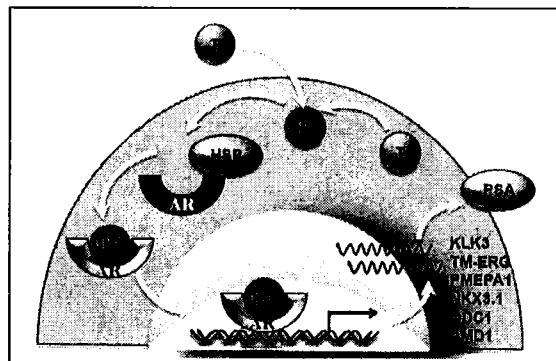
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## INTRODUCTION

Although most prostate cancers are initially responsive to androgen ablation therapy, they become treatment resistant as tumor cells develop mechanisms to evade the treatment. Multiple mechanisms invoking gain of AR signaling (increased intra-tumoral androgen bio-synthesis, elevated AR function, ligand independent splice variants, and the recently described AR activating long non-coding RNAs (Egan, Dong et al. 2013; Yang, Lin et al. 2013) or loss of AR signaling (activation of AR independent survival pathways) can lead to androgen ablation refractory or Castration Resistant Prostate Cancer (CRPC). Dysfunctions (both gain and loss) of the male hormone receptor, the androgen receptor (AR) mediated signaling play important roles in CaP development or progression (Richter, Srivastava et al. 2007) (Ryan and Tindall 2011; Dobi, Sreenath et al. 2013).

Although AR expression can be detected throughout prostate tumorigenesis, the diagnostic or prognostic utility of monitoring AR levels has been challenging. Studies evaluating the association of AR protein levels with poorly differentiated tumors, higher Gleason score or with decreased PSA recurrence-free survival are inconclusive (Romics, Banfi et al. 2008), (Qiu, Leuschner et al. 2008), (Wako, Kawasaki et al. 2008), (Szabo, Bartok et al. 2009) (Szabo et al. 2009). **We reasoned that evaluation of AR dysfunction may be predictive of poor outcome and androgen ablation therapy failure.** The proposed approach would significantly impact the patient stratification for new emerging therapeutic strategies targeting the androgen axis (Mitsiades 2013) (Azzouni and Mohler 2012).

Novel insights into the androgen regulated transcriptome have provided by several prostate cancer research laboratories, including ours. These endeavors identified direct transcriptional regulatory targets of AR which have promise in defining the role of AR dysfunctions, as well as, in providing novel, functionally relevant biomarkers and potential therapeutic targets (Xu, Srikantan et al. 2000), (Segawa, Nau et al. 2002), (Velasco, Gillis et al. 2004), (Dehm and Tindall 2006), (Wang, Li et al. 2007) (Richter, Srivastava et al. 2007), (Heemers and Tindall 2009), (Li, Xu et al. 2008) (Li et al 2008), (Wang, Li et al. 2009), (Mostaghel, Geng et al. 2010). Previous, as well as, recent studies revealed androgen regulation of *ERG* (*TMPRSS2-ERG*), *KLK3*(PSA), *NKX3.1*, *PMEPA1*, *AMD1* and *ODC1* genes in prostate cancer cells (Xu, Shanmugam et al. 2000; Segawa, Nau et al. 2002;



**Figure 1. AR can be altered via numerous mechanisms, however the net effect of these changes is reflected in defective transcription factor functions of the AR.**

Masuda, Werner et al. 2005; Tomlins, Rhodes et al. 2005; Mostaghel, Geng et al. 2010) (Figure 1). Decreased expression of androgen regulated genes in association with attenuated response to 5 $\alpha$ -reductase inhibition in benign prostate tissues has also been reported (Mostaghel, Geng et al. 2010). The goal of these studies is to provide early warning signs of androgen independence and to predict the success or failure of treatment response. Data obtained from prostate cancer cell culture models highlighted a distinct AR regulated transcription program in androgen blockade resistant derivatives of LNCaP cells (Wang, Li et al. 2009).

We have reported an overall decrease in androgen regulated gene expression with prostate cancer progression (Sterbis, Gao et al. 2008). Other reports have also noted a signature of attenuated AR function in late stage, especially in metastatic prostate cancer in human specimens (Varambally, Yu et al. 2005), (Hermans, van Marion et al. 2006), (Tomlins, Mehra et al. 2007), (Mendiratta, Mostaghel et al. 2009), as well as in a xenograft models (Hendriksen, Dits et al. 2006). During the evaluation of an AR regulated gene panel in selected tumor specimens we reported the association of attenuated AR regulated gene expression with poorly differentiated tumor phenotype at the time of radical prostatectomy and with biochemical recurrence (Dobi, Furusato et al. 2010).

## **BODY**

**To develop readouts for AR function in CaP cells, this DoD/CDMRP grant award focused on the quantitative measurements of AR regulated genes in carefully isolated benign and tumor cells and in prostate tumor tissues.**

**The hypothesis of this proposal is that the *in vivo* status of AR function in prostate tumor cells can be precisely defined by measuring the expression of a panel of AR regulated genes (*KLK3/PSA*, *PMEPA1*, *TMPRSS2-ERG*(*ERG*), *NKX3.1*, *AMD1* and *ODCI*). The objective of this proposal is to predict the course of prostate cancer progression by monitoring a panel of AR regulated genes in stratifying patients for treatment modalities.**

The findings reported here reflect the progress of the first and second years of the proposal towards the evaluation of androgen receptor function in towards the prognosis and therapeutic stratification of prostate cancers.

**Aim #1: To establish the AR regulated gene panel (ARP) as a quantitative measure of *in vivo* AR functional status in prostate cancer at the time of radical prostatectomy.**

*Tasks to perform:*

**Task 1: to establish the AR regulated gene panel (ARP) as a quantitative measure of *in vivo* AR functional status in prostate cancer at the time of radical prostatectomy.**

**Step 1 (Months 1-6):**

Institutional Review Board regulatory review and approval processes will occur at two separate institutions: the Walter Reed Army Medical Center (WRNMMC) and the Uniformed Services University of the Health Sciences (USUHS).

During the IRB approval process probes and primers will be designed following the same principles we have been using for TaqMan and will be tested for specificity and sensitivity by using copy number defined dilutions of cDNA clones of ARP.

**Step 2 (Months 6-22):**

RNA samples for QRT-PCR will be obtained from radical prostatectomy specimens of 110 CaP patients following our established strategy. Total RNA from laser-captured microdissected (LCM) normal and cancer cells from either formalin fixed paraffin embedded (FFPE) or OCT embedded and H&E stained frozen prostate sections of radical prostatectomy specimens (5-10,000 epithelial cells per sample) will be acquired from the CPDR Biospecimen Bank. Total RNA will be quantified by using Ribogreen fluorometric method. The total RNA isolated from the paired tumor and normal LCM epithelium specimens will be converted to cDNA. The expression levels of ARP genes (*PSA/KLK3*, *PMEPA1*, *NKX3.1*, *ODC1*, *AMD1* and *TMPRSS2-ERG*) will be determined in the matched tumor and normal prostate tissue-derived cDNA samples by real time QRT-PCR (TaqMan). The expression of *GAPDH* will be simultaneously analyzed as endogenous control.

#### Step 3 (Months 23-24):

Gene AR panel expression data will be analyzed by informatic and statistical methods for positive or negative correlation with aggressiveness of prostate cancer, as defined by Gleason grade, pathological stage, biochemical recurrence and for feasibility of gene expression measurement in a clinical assay (specificity, sensitivity and reproducibility). Cumulative index will be used for quantitative definition of AR function (ARF index) towards determine the stratification power of AR gene panel at the time of radical prostatectomy. This index will be incorporated into nomograms modeling time-to-event data, including prediction of disease progression, combined with established clinical and pathological characteristics that predict this study endpoint.

#### Accomplished

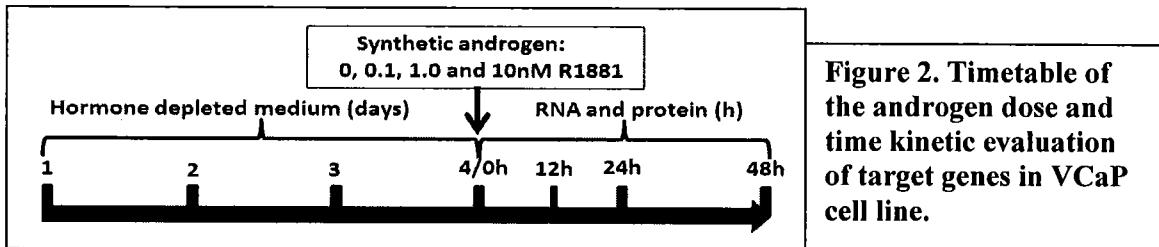
#### Step 1 (Months 1-6):

- Institutional Review Board regulatory review and approval was obtained from two separate institutions: the Walter Reed Army Medical Center (WRAMC) and the Uniformed Services University of the Health Sciences (USUHS).
- The primers and probes were designed for TaqMan assay of *PSA/KLK3*, *PMEPA1*, *NKX3.1*, *ODC1*, *AMD1* and *TMPRSS2-ERG* genes, quality control has been completed and probes has been tested for specificity and sensitivity.

#### Novel Findings

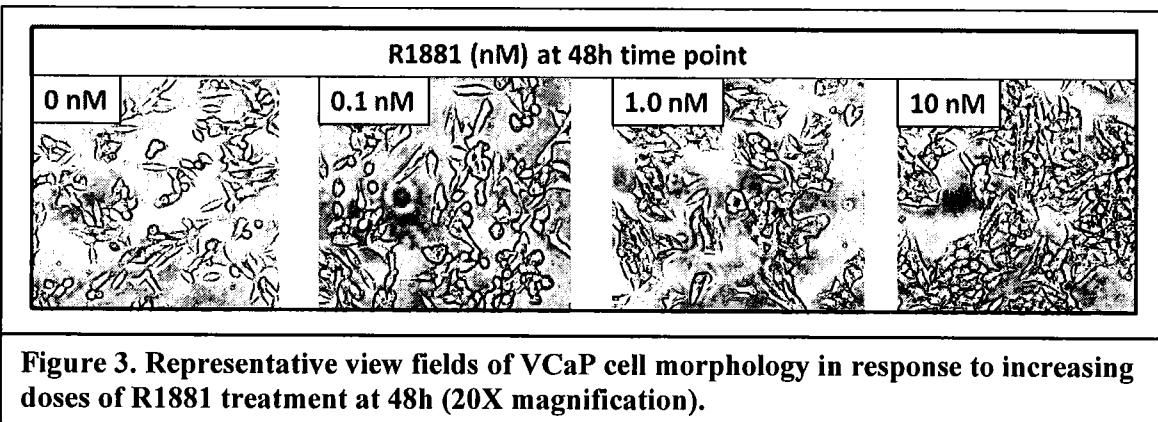
To monitor the functional status of androgen receptor in prostate tumors a panel of six androgen inducible genes were selected. This panel includes tissue (*KLK3/PSA*), AR stability regulator (*PMEPA1*), transcription factor (*NKX3.1*), polyamine biosynthesis (*ODC1*, *AMD1*) and oncogene (*TMPRSS2-ERG*) protein coding genes. Selected genes are either direct targets of AR or are tightly regulated by AR and have demonstrated prostate associated expression encompassing major biological functions regulated by AR in the human prostate. The primers and probes have been obtained, and tested by using cDNA from VCaP prostate cancer cell line endogenously expressing all target genes, *PMEPA1* (Locus ID: GXL\_128240), *TMPRSS2-ERG* fusion A (Locus ID: GXL\_39091), *KLK3/PSA* (LocusID: GXL\_32002), *NKX3.1* (LocusID: GXL\_29827), *AMD1* (Locus ID: GLX\_261249, and *ODC* (Locus ID: GXL\_75806). PCR amplicons were isolated and target regions were confirmed by DNA sequencing.

To assess the performance of primers and probes assessing androgen dose and time kinetic response of target genes, VCaP cells were grown in androgen depleted conditions for four days. To induce androgen regulated genes, synthetic androgen (R1881) was added to the cell cultures at 0, 0.1, 1.0 and 10nM concentrations and cells were harvested at 0, 12, 24 and 48h time points. Cell morphology in response to R1881 treatment was monitored by microscopy (Figure 2.).



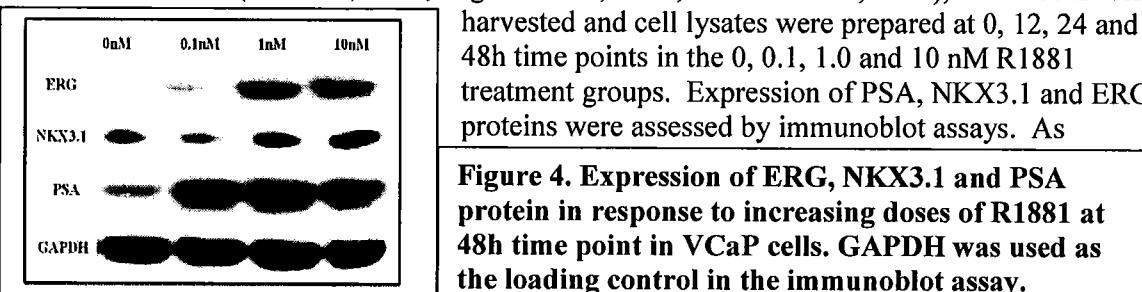
**Figure 2. Timetable of the androgen dose and time kinetic evaluation of target genes in VCaP cell line.**

To monitor changes in cell morphology in response to R1881 induction, cell were assessed at 0, 12, 24 and 48h time points. The observed cell morphology was consistent with the expected time and dose kinetic response of VCaP cells to R1881 treatment (Figure 3.).



**Figure 3. Representative view fields of VCaP cell morphology in response to increasing doses of R1881 treatment at 48h (20X magnification).**

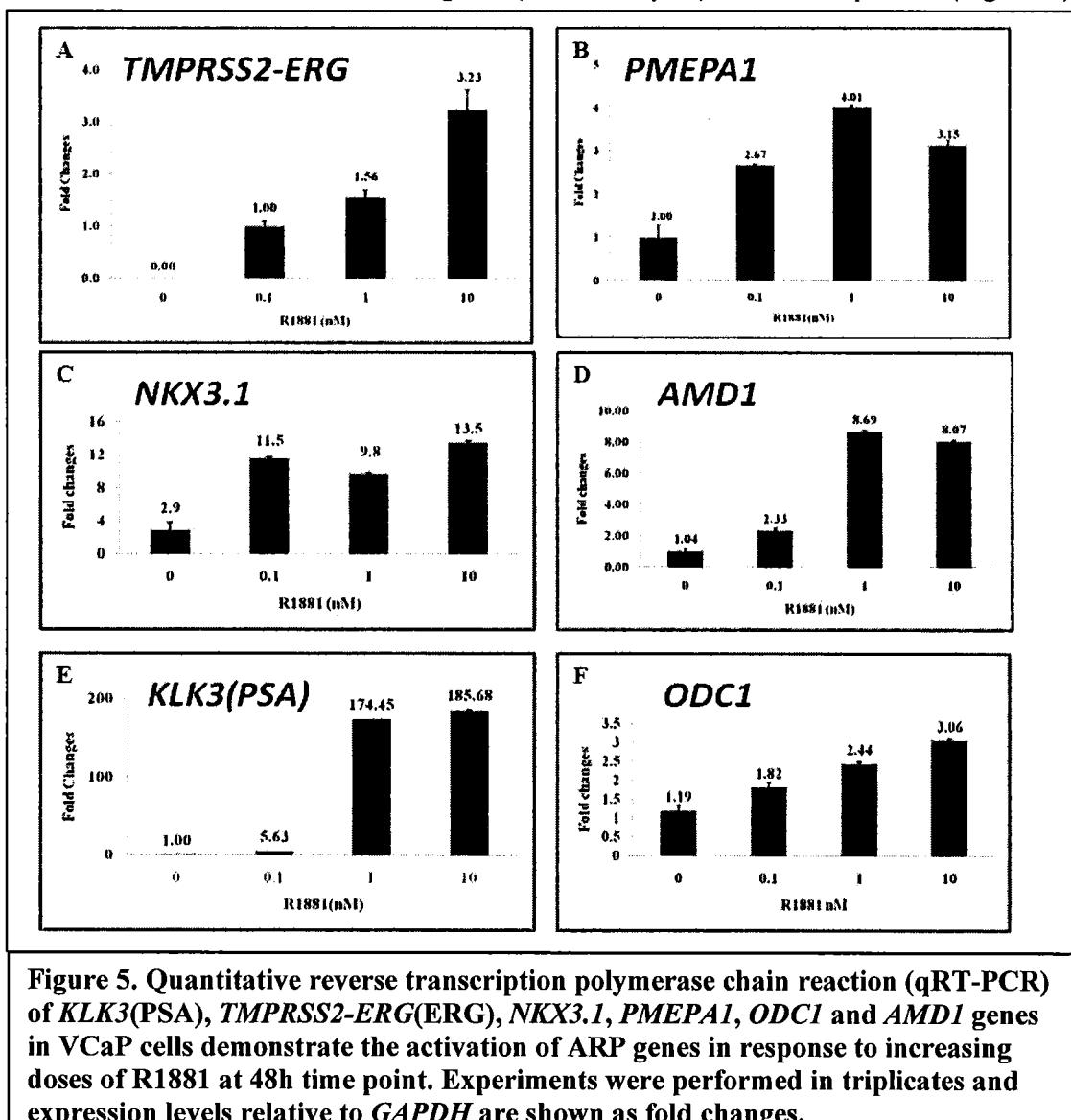
To evaluate protein products of three of the target genes (PSA, NKX3.1 and ERG) that was previously shown robust response to androgen induction in hormone responsive prostate cancer cell culture models (Xu et al., 2001; Segawa et al., 2002; Tomlins et al., 2005), VCaP cells were harvested and cell lysates were prepared at 0, 12, 24 and 48h time points in the 0, 0.1, 1.0 and 10 nM R1881 treatment groups. Expression of PSA, NKX3.1 and ERG proteins were assessed by immunoblot assays. As



**Figure 4. Expression of ERG, NKX3.1 and PSA protein in response to increasing doses of R1881 at 48h time point in VCaP cells. GAPDH was used as the loading control in the immunoblot assay.**

expected, the experiment revealed androgen hormone dose dependent expression of the assayed proteins (Figure 4.).

For the evaluation of ARP target gene expressions RNA was isolated from VCaP cells at 0, 12, 24 and 48h in the 0, 0.1, 1.0 and 10 nM R1881 treatment groups. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in triplicates and one control reaction without RT were performed for each RNA sample by using primer and probe sets for *KLK3*(PSA), *TPMRSS2-ERG*(fusion junction “A” (Tomlins *et al.*, 2005)), *NKX3.1*, *PMEPA1*, *ODC1* and *AMDI* genes and expression values were expressed relative to *GAPDH*. Expression results were calculated from the average  $C_T$  (threshold cycle) values of triplicates (Figure 5.).



Specific primers and probes were designed for the qRT-PCR analyses of *PSA/KLK3*, *PMEPA1*, *NKX3.1*, *ODC1*, *AMDI* and *TPMRSS2-ERG* genes. Quality control of primer and probe sets has been completed. Kinetic and androgen dose response of endogenously

**expressed levels of ARP genes in VCaP cells indicate the sensitivity of detection. Sequence analyses of target gene amplicons confirmed the specificity of detecting ARP genes.**

**Step 2 (Months 6-22):**

RNA samples for QRT-PCR were obtained from radical prostatectomy specimens of 77 CaP patients following our established strategy. Total RNA from laser-captured microdissected (LCM) normal and matching cancer cells from OCT embedded and H&E stained frozen prostate sections of radical prostatectomy specimens (5-10,000 epithelial cells per sample) were acquired from the CPDR Biospecimen Bank. Total RNA was determined by using RiboGreen fluorometric method. The total RNA isolated from the paired tumor and normal LCM epithelium specimens were converted to cDNA. The expression levels of ARP genes (*PSA/KLK3*, *PMEPA1*, *ERG*, *TMPRSS2-ERG*) were determined in the matched tumor and normal prostate tissue-derived cDNA samples by real time QRT-PCR (TaqMan). The expression of *GAPDH* was simultaneously analyzed as endogenous control.

**Novel Findings**

Radical prostatectomy specimens were obtained from patients enrolled in the Center for Prostate Disease Research (CPDR) program from 1996 to 2010. Clinico-pathologic data were obtained from the CPDR database. Optimum cutting temperature (OCT) embedded RP tissues specimens from 77 patients were analyzed in this study (Table 1). None of these patients had received androgen deprivation therapy. Biochemical recurrence was defined as two consecutive post-operative PSA values greater than 0.2 ng/mL measured at  $\geq 8$  weeks post-operatively. Laser capture microdissection (LCM) derived cells were obtained from OCT embedded frozen sections. Specimens obtained immediately after surgical resection were OCT embedded on glass slides, frozen on dry ice and stored at  $-80^{\circ}\text{C}$ . Benign and malignant cells were isolated by LCM using 5 micron frozen tissue sections. Total RNA from the LCM derived specimens was isolated, purified and quantified.

In the interim analyses of ARP genes we carefully examined tumor and matched normal samples from Caucasian American (CA) and African American (AA) patients due to reported differences

Race	Caucasian American	African American
<b>Number of patients</b>	42	35
<b>Gleason Sum</b>		
6	17(41.5)	14(41.2)
7	15(36.6)	16(47.1)
8 to 10	9(22)	4(11.8)
<b>Pathologic T stage</b>		
pT2	17(42.5)	14(41.2)
pT3-4	23(57.5)	20(58.8)
<b>Surgical margin status</b>		
Negative	39(92.9)	28(82.4)
Positive	3(7.1)	6(17.6)
<b>LCM differentiation</b>		
Well	28(71.8)	30(93.8)
Poorly	11(28.2)	2(6.3)
<b>PSA at diagnosis</b>		
Mean (SD)	6.3(4.4)	11.2(16.5)
Median (range)	5.2(1.1,23.4)	6.6(3.2,98.7)
<b>Seminal vesicle invasion</b>		
Negative	39(92.9)	28(82.4)
Positive	3(7.1)	6(17.6)
<b>Extra capsular extension</b>		
Negative	14(51.9)	9(33.3)
Positive	13(48.1)	18(66.7)
<b>Age at RP</b>		
Mean (SD)	61.1(8.4)	59.4(8.3)
Median (range)	62.1(40.2,73.6)	59.8(45,71.9)
<b>BMI</b>		
Mean (SD)	26.2(3.5)	27.3(4.9)
Median (range)	26(20,34)	28(19,42)

**Table 1. Clinico-pathologic characteristics**

in incidence and mortality (Table 1). The analysis showed positive correlation between the expressions of ARP genes (*ERG* vs. *PMEPA1* and *ERG* vs. *PSA*) in tumors of AA patients that has not observed within the CA group (Table 2). In the assays *GAPDH* expression was used as the control.

Gene matrix		CA			AA		
		N	Pho	P value	N	Pho	P value
ERG T ^ Ct	PMEPA1 T ^ Ct	30	-0.03	0.8896	24	0.57	<b>0.0033</b>
ERG T ^ Ct	PSA T ^ Ct	28	0.06	0.7398	24	0.46	<b>0.0220</b>
PMEPA1 T ^ Ct	PSA T ^ Ct	39	0.01	0.9522	35	0.17	0.3386

**Table 2. Positive correlation between the expressions of ERG vs. PMEPA1 and ERG vs. PSA expression in tumors of AA patients.**

Within the currently examined cases (n=77) decreased *PMEPA1*, *PSA* or *ERG* expression showed no correlation with Pathological T-stage or Gleason sum. Indeed, the power calculation of this proposal indicated that n=110 will be necessary to reach sufficient statistical power for the evaluation of the cumulative ARP index. We are continuing to monitor the correlation of ARP genes with biochemical recurrence and metastasis. Furthermore, we will include in the cumulative index the qRT-PCR results of *NKX3.1*, *ODC1* and *AMD1* data that will be completed within the next reporting period.

#### **Aim #2: to define the utility of ARP proteins in monitoring the AR function.**

*Tasks to perform:*

##### **Task 2: Define the utility of ARP proteins in monitoring the AR function.**

###### **Step 1 (Months 12-30):**

IHC will be set up and optimized with antibodies against ARP gene products. Whole-mounted sections of RP specimens with prostate cancer will be assayed in a cohort of over 110 patients by immunohistochemistry. The staining intensities will be determined according to percent of cells positive. The intensity will be scored and a combination of measurements will be calculated by multiplying the percent of positive cells with the degree of intensity, which will result in an IHC intensity score. The sum of staining intensity scores will be expressed as the cumulative IHC staining index of AR regulated gene panel.

###### **Step 2 (Months 12-30):**

To establish concordance between the expression of ARP mRNA and proteins branched-chain DNA (b-DNA) signal amplification method will be used. Adjacent 4  $\mu$ m-thick section will be selected from each of the FFPE whole-mount prostate samples.

###### **Step 3 (Months 31-36):**

Incorporate the AR gene panel cumulative indexes into predictive nomograms for prostate cancer progression defining predictive power and utility for patient stratification.

Translational products of the AR regulated gene panel will be assessed in formalin fixed paraffin embedded (FFPE) tissues by evaluating 110 whole-mounted radical prostatectomy specimens. To address the concordance between mRNA and protein expression adjacent sections will be analyzed by the recently developed bDNA technology. From the IHC staining of products of the ARP will be summarized in a cumulative index for patient stratification. IHC scores and a cumulative pathology scores will be established for the tumor foci in the sections. Cumulative IHC score will be evaluated alone and by combining with nomograms modeling time-to-event data incorporating the biochemical recurrence within eight years of follow up. Post-operative predictive value of existing, validated nomograms will be assessed by using the patient cohort. The IHC-derived ARP values, as a single cumulative index, will be incorporated into validated nomograms (Kattan) modeling time-to-event data, including prediction of CaP progression, combined with established clinical and pathological characteristics that predict this study endpoint. The concordance index, C, will be used to assess the improvement in model fit upon inclusion of AR function index.

### **Accomplished**

#### **Step 1** (Months 6-12):

- The Whole-mounted sections of RP specimens with prostate cancer have been identified in a cohort of over 110 patients for immunohistochemistry. We have focused our initial Immunohistochemical assessment of ARP genes on the patient cohort that we have previously defined for ARP gene defects in microarray-based gene expression studies (Dobi et al., 2010). IHC has been completed for 40 cases for ERG, PSA, NKX3.1 and AR. The staining intensities have been determined according to percent of cells positive. The intensity was scored and a combination of measurements was calculated by multiplying the percent of positive cells with the degree of intensity (IHC intensity score).

#### **Step 2** (Months 9-12):

To establish concordance between the expression of ARP mRNA and proteins we have performed a direct comparison of ARP IHC results to mRNA expression data from index tumors of the same patients in this 40 prostate cancer cases.

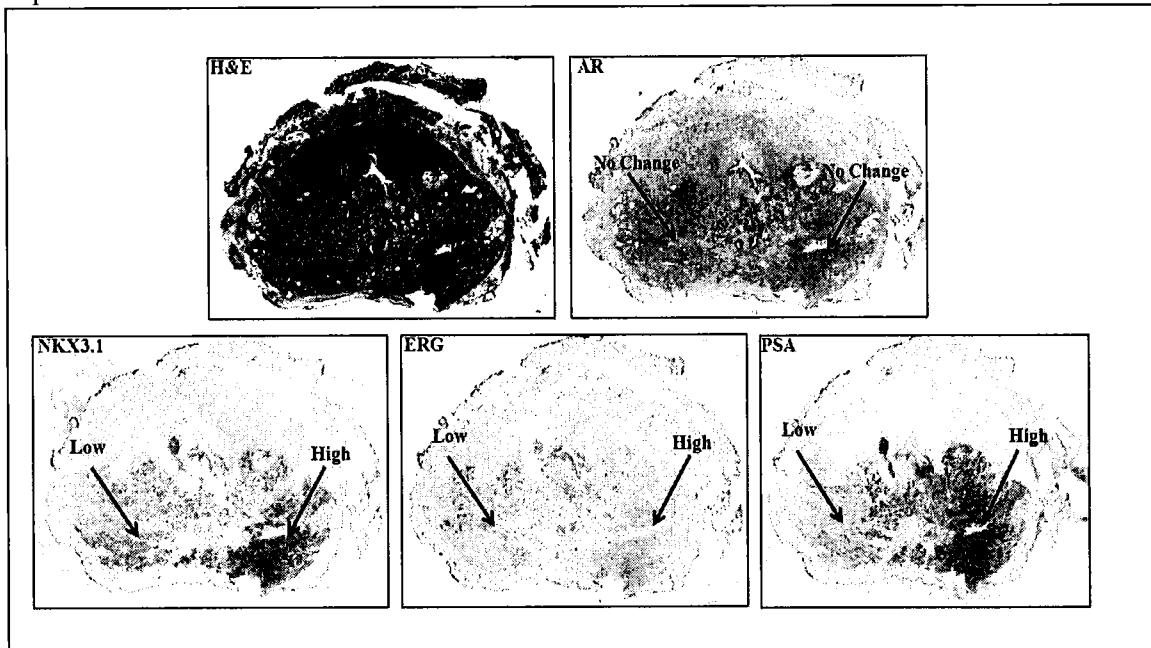
#### **Step 3** (Months 31-36):

As an initial assessment of predictive performance of protein products of the AR regulated genes formalin fixed paraffin embedded (FFPE) tissues of 40 whole-mounted radical prostatectomy specimens were examined. To address the concordance between mRNA and protein expression index tumors of adjacent sections were analyzed for IHC and compared to gene expression data obtained from frozen OCT embedded ex vivo biopsy specimens. IHC staining scores of ARP proteins and RNA expression were summarized in cumulative indexes and were compared to biochemical recurrence and metastasis.

### **Novel Findings**

Radical prostatectomy specimens were analyzed for ARP in 40 cases that were previously fixed in 10% buffered formalin and embedded as whole mounts in paraffin. Each prostate was sectioned at 0.22 cm intervals in a transverse plane perpendicular to the long axis of the posterior

surface of the prostate and completely embedded as whole mounts. IHC staining on adjacent four-micron sections of the whole-mounted blocks were performed by using mouse monoclonal anti-ERG antibody developed by our laboratory (CPDR ERG-MAb, (Furusato, Tan et al. 2010), (Mohamed, Tan et al. 2010), (Miettinen, Wang et al. 2011) (Braun, Goltz et al. 2011) (Rosen, Sesterhenn et al. 2012), rabbit polyclonal anti-PSA antibody (DAKO, A056201-2), rabbit polyclonal anti-NKX3.1 antibody (a kind gift from Dr. Charles Biebrich, UMBC) and mouse monoclonal anti-AR antibody (SantaCruz, sc-7305) (Figure 6.). Slides were incubated with these antibodies and were counterstained with hematoxylin. The protein expression was assessed based on both the amount and intensity of immunopositive cells. Intensities were scored from 0 to 2 as follows: 0 as negative, 1 as weak, and 2 as strong. The percentage of positively stained area was estimated and scored from 1 to 4 as follows: less than 25% positive area as score 1, 25-50% score 2, 51-75% score 3, and more than 75% positive area score 4. The final score was determined after multiplying the intensity score and percentage of positively stained area in the respective lesions.

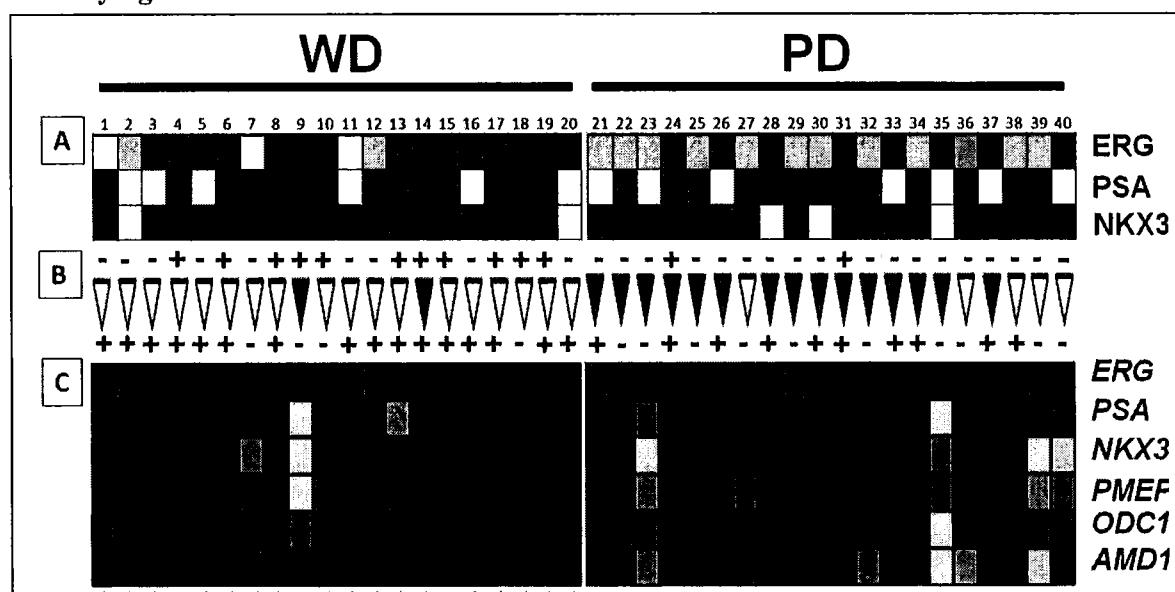


**Figure 6.** Evaluation of whole-mount prostate sections for the expression of ARP genes by immunohistochemistry. On the upper left panel two tumor foci (T1 and T2) are shown by H&E. The upper right panel shows equal AR immunostaining in both tumor foci (no change). Lower panels show reduced immunoreactivity of NKX3.1, ERG and PSA in the left tumor focus (Low), in contrast to the tumor focus shown at the right side (High) of the whole-mount prostate section.

**Assessment of AR dysfunction by the IHC evaluation of ARP genes in whole mount prostate cancer specimens indicates that in a sub-set of cases tumor foci can be identified with reduced expression of ARP genes supporting the central hypothesis of the proposal that AR function in prostate tumor cells can be defined by measuring the expression of a panel of AR regulated genes.** From the IHC assessment index tumors were classified based on ARP by the cumulative IHC index of “-” when one or more of the ARP protein expression was lost or decreased (Figure 7). We have plotted the ARP scores on the biochemical recurrence (BCR) and metastasis data. IHC staining for AR was uniform, and as expected, it was non-informative. Thus, we excluded AR from further analyses.

Towards the goals of Step 2 in Aim 2, to establish concordance between the expression of ARP mRNA and proteins we compared the IHC values to the gene expression data of the ARP panel (cumulative index  $CI > 0$  marks intact AR function “+” or  $CI \leq 0$  marks defects of AR function “-”).

**IHC assessment of AR dysfunction by monitoring ERG, NKX3.1 and PSA proteins shows remarkable accuracy in identifying BRC and metastasis in tumors with PD morphology (14 out of 15 BRC/metastasis). In contrast, intact AR function confirmed by detecting RNA levels of ARP genes performs better in predicting favorable outcome in tumors with WD morphology, precisely identifying 13 cases with no BRC and no metastasis out of 14 cases classified as intact AR.**



**Figure 7.** (A) Attenuated expression are frequently (85%) found in tumors with poorly-differentiated (PD, patient 21-40) morphology as indicated by the IHC heatmap of index tumors in 40 prostate cancer cases (Red: IHC positive (95%-100% & 2-3+); Yellow: intermediate, 1-2+); Green: negative). (B) BRC (red and black triangles) and metastasis (black triangles), no recurrence (white triangles) or no follow up data (yellow triangles) symbols are aligned with the cumulative ARP index on top (“+” marks intact AR signaling; “-“ indicates that at least one of the AR readout proteins in the panel is absent or attenuated in IHC. (C) Gene expression (GeneChip) data of the corresponding index tumors indicates elevated (red) decreased (green) or unchanged (black) expression values of the AR regulated genes. According to the cumulative index definition AR signaling is considered compromised and marked as “-“ when the expression of three or more AR regulated genes are reduced ( $CI \leq 0$ , Dobi et al., TOCJ, 2010).

Encouraged by the promising data observed by the analysis of ARP proteins (Figure 7.) in the second reporting period we have completed a collaborative study with Dr. Lukas Bubendorf, University of Basel directly addressing the correlation of ERG protein expression (as the result of androgenic activations) and ERG gene rearrangements on the progression to castration resistant prostate cancer (CRPC). In this study design we examined tissue microarray from 114 hormone naïve and 117 CRPCs. We analyzed the expression of ERG oncoprotein by IHC and ERG rearrangement status by fluorescence in-situ hybridization. Also, we monitored the protein expression levels of AR and the proliferation marker Ki67. Consistent with previous reports the *TMPRSS2-ERG* gene fusion status showed correlation with the presence or absence of ERG protein both in hormone naïve and in CRPC specimens ( $p<0.0001$ ). **The major finding of the study is the complete absence of ERG protein in 26% of CRPC cases harboring ERG genomic rearrangement. Moreover, this subset showed significantly lower levels of AR protein expression providing a strong support to the central hypothesis of the proposal.**

## KEY RESEARCH ACCOMPLISHMENTS

### **Aim #1: To establish the AR regulated gene panel (ARP) as a quantitative measure of *in vivo* AR functional status in prostate cancer at the time of radical prostatectomy.**

- Within the first reporting period Towards Institutional Review Board approval was obtained from the Walter Reed National Military Medical Center (WRNMMC) and from the Uniformed Services University of the Health Sciences (USUHS).
- The primers and probes were prepared and QC-ed for qRT-PCR assays for assessing the expression of *PSA/KLK3*, *PMEPA1*, *NKX3.1*, *ODC1*, *AMD1* and *TMPRSS2-ERG* genes. The quality control of primers and probes for the detection of endogenously expressed ARP genes has been completed, target sequences were confirmed by DNA sequencing of PCR products and sensitivity and specificity was confirmed.
- In the second reporting period RNA samples for QRT-PCR were obtained from radical prostatectomy specimens of 77 CaP patients. Total RNA from laser-captured microdissected (LCM) normal and matching cancer cells from OCT embedded and H&E stained frozen prostate sections of radical prostatectomy specimens were acquired from the CPDR Biospecimen Bank. The total RNA isolated from paired tumor and normal LCM epithelium specimens. The expression levels of ARP genes (*PSA/KLK3*, *PMEPA1*, *ERG* and *GAPDH*) were determined in matched tumor and normal prostate tissue-derived cDNA samples by real time qRT-PCR (TaqMan).

### **Aim #2: to define the utility of ARP proteins in monitoring the AR function.**

- Whole-mounted sections of RP specimens with prostate cancer have been identified in a cohort of 110 patients for the Immunohistochemical assessment of ARP proteins.

- To establish concordance between the expression of ARP mRNA and proteins we have completed the direct comparison of ARP IHC results to mRNA expression data from index tumors of the same patients in 40 prostate cancer cases. The result showed 90% concordance between detecting ERG protein or ERG mRNA validating the identical origin of tumor samples assessed by IHC and by gene expression experiments. However, the overall concordance between the cumulative indexes of ARP proteins and genes were only 50%. This finding is due to a somewhat unexpected discordance between the protein and mRNA levels of the NKX3.1 and PSA (KLK3) genes.
- Within the second reporting period IHC has been completed for 40 cases for ERG, PSA, NKX3.1 and AR. The staining intensities have been determined and intensity was scored and a combination of measurements was calculated. Assessment of ARP genes by IHC showed remarkable accuracy in identifying BRC and metastasis in tumors with PD morphology (14 out of 15 BRC/metastasis). In contrast, detection of RNA levels of ARP genes performs better in predicting favorable outcome in tumors with WD morphology, precisely identifying 13 cases with no BRC and no metastasis out of 14 cases classified as intact AR. In a collaborative study we have examined the correlation between AR and ERG expression comparing to the *ERG* genomic rearrangement status in tissue microarray from 114 hormone naïve and 117 CRPCs. A subset of *ERG* rearranged tumors without detectable ERG protein and absent or decreased AR was identified in 26% of CRPC cases indicating dispensed AR (manuscript in review).

## REPORTABLE OUTCOMES

### Publications

Dobi A, Sreenath T, Srivastava S.: Biological and Clinical Implications Androgen-responsive genes in prostate cancer: Regulation function and clinical application (invited article edited by Zhou Wang), Chapter 19: Androgen-dependent oncogenic activation of ETS transcription factors by recurrent gene fusions in prostate cancer: Springer Science & Business Media, LLC2013, BWF Book 300271, New York USA DOI 10.1007/978-1-4614-6182-1\_19 (2013).

Abstract half of all prostate cancers in the Western countries harbor gene fusions that involve regulatory sequences of the androgen receptor (AR)-responsive genes (predominantly TMPRSS2) and protein coding sequences of nuclear transcription factors of the ETS gene family (predominantly ERG). This leads to unscheduled androgen-dependent expression of ETS-related transcription factors in tumor cell-specific manner. Extensive evaluations of ERG alterations at genome, transcript, and protein levels demonstrate unprecedented specificity of ERG for detecting prostate tumor cells. Assessment of ERG alterations in combination with other common prostate cancer gene alterations (*AMACR*, *PCA3*, *p63*) has potential in improving CaP diagnosis. Utility of ERG in assessing the clinical behavior of prostate cancer is uncertain. **Strong correlation of ERG expression with known androgen-responsive genes in prostate tumors has potential in developing gene panels inclusive of ERG for monitoring androgen receptor functional status in the disease continuum.** Studies focusing on oncogenic functions of ERG point to its involvement in: abrogating differentiation; facilitating cell invasion and epithelial to mesenchymal transition; and disrupting epigenetic, inflammatory, and DNA

damage control mechanisms. Therapeutic targeting of ERG or ERG interacting proteins, such as PARP hold promise in developing new strategies for the treatment of prostate cancer. In summary multipronged evaluations of the ERG in CaP continue to reflect the critical role of this prevalent oncogenic activation in a CaP.

### **Poster presentations**

#### **1) American Association for Cancer Research (AACR) Annual Meeting April 6-10, 2013, Washington, DC**

Albert Dobi, Denise Young, Wei Huang, Lakshmi Ravindranath, Shashwat Sharad, Hua Li, Gyorgy Petrovics, David G. McLeod, Isabell A. Sesterhenn and Shiv Srivastava: Evaluation of the Androgen Receptor Function Index (ARFI) in Prostate Cancer

**Introduction:** Initially prostate cancer is driven by the male hormone androgen through the androgen receptor (AR). However, in some cases androgen receptor is functionally altered at late stages of tumorigenesis. Early knowledge of androgen receptor dysfunctions may assist in patient stratification for emerging therapeutic strategies. Although AR function can be altered by numerous mechanisms, the net effect of these changes is reflected in defective transcription factor functions of the AR. To develop readouts for AR function in prostate cancer we have evaluated the expression of a functionally relevant panel of AR-regulated genes in a prostate cancer cell culture model and in whole-mounted prostate tumor tissues.

**Methods:** In the VCaP cell culture model of prostate cancer that endogenously express, AR, TMPRSS2-ERG fusion (ERG), KLK3 (PSA), NKX3.1, PMEPA1, ODC1 and AMD1 genes we have evaluated the dose and time kinetics of these AR regulated genes in response to androgen (R1881) treatment at protein and mRNA levels. Radical prostatectomy specimens representing favorable or poor prognostic cases were selected for the study. Each prostate was fixed, sectioned and immunohistochemical (IHC) staining on adjacent four-micron sections of the whole-mounted blocks were performed by using mouse monoclonal anti-AR, anti-ERG antibody (clone 9FY), rabbit polyclonal anti-PSA and anti-NKX3.1 antibodies. IHC intensities were scored and the final score was determined after multiplying the intensity score and percentage of positively stained area in the respective lesions.

**Results:** Towards defining the expression levels of AR regulated genes in prostate cancer cells we have evaluated and confirmed the androgen dose and time kinetic response of endogenously expressed KLK3(PSA), PMEPA1, NKX3.1, ODC1, AMD1 and TMPRSS2-ERG (ERG) genes in VCaP cell culture model. Assessment of AR dysfunction by the immunohistochemical evaluation of AR regulated genes in whole-mounted prostate cancer specimens showed reduced expression of AR regulated genes in a sub-set of prostate cancer cases with notable inter- and intratumoral heterogeneity.

**Conclusions:** Androgen regulation of examined genes was confirmed in VCaP prostate cancer cell culture model. Assessment of AR, ERG, NKX3.1 and PSA protein levels by IHC indicated reduced expression of AR-regulated genes in a subset of the cases. The analysis revealed inter-

and intratumoral heterogeneity, as well as, challenges in the quantitative assessment of IHC. **The observed readouts of AR dysfunction may provide a new tool for improved prognostic accuracy and patient stratification at early stages of prostate cancer treatment.**

Funding: This work was supported by the Department of Defense, Prostate Cancer Research Program, (PCRP) of the Office of the Congressionally Directed Medical Research Programs (PC100700) to SS and AD.

**2) 8th Early Detection Research Network (EDRN) Scientific Workshop  
March 13-15, Bethesda, MD**

Albert Dobi, Gyorgy Petrovics, Denise Young, Wei Huang, Lakshmi Ravindranath, Shashwat Sharad, Yongmei Chen, Isabell A. Sesterhenn, David G. McLeod and Shiv Srivastava:  
Evaluation of the Functional Status of Androgen Receptor in Prostate Cancer

**Introduction & Objectives:** Prostate cancer development is initially driven by the male hormone androgen through the androgen receptor (AR). Inhibition of AR and the associated hormone signaling pathway is the cornerstone for therapy of late stage prostate cancer. Although, AR expression can be detected throughout the continuum of tumor progression, it is heterogeneous and changes over time. Thus, AR expression levels do not seem to be a useful marker for differentiating indolent from aggressive prostate tumors. Indeed, there is an apparent discordance between AR levels and AR function during clonal selection and progression of prostate cancer. Thus, early knowledge of the functional status of androgen receptor may help in identifying aggressive tumor phenotypes. We have been developing an Androgen Receptor Function Index (ARFI) for monitoring the functional status of AR by measuring the expression of androgen regulated genes.

**Materials & Methods:** Expression of AR regulated KLK3(PSA), PMEPA1, NKX3.1, ODC1, AMD1 and TMPRSS2-ERG(ERG) genes were evaluated in a GeneChip dataset obtained from a panel of laser capture micro-dissected well/moderately differentiated (WD) (n=20) and poorly differentiated (PD) (n=20) tumor cells derived from primary tumors from 40 patients, who had no prior androgen ablation treatment. Whole-mounted sections of radical prostatectomy specimens from the same group of patients were evaluated by Immunohistochemistry assessing of AR, ERG, NKX3.1 and PSA protein levels.

**Results:** The mRNA expression of evaluated AR regulated genes showed consistent AR dysregulation patterns. Remarkably, low ARFI expressing cases were highly enriched in tumors with PD morphology (80%). In contrast, only 30% of WD tumors were noted in low ARFI expressing cases. Immunohistochemical assessment of ERG, NKX3.1 and PSA proteins indicated reduced expression of ARFI proteins in distinct tumor foci within a subset of the cases.

**Conclusions:** **These findings suggest that integrating the expression levels of AR target genes in a cumulative index (ARFI) may be a useful approach for defining subdued AR function with future potential in stratifying patients for targeted therapy on the basis of overall AR functional status in primary tumors.**

Acknowledgement: This study was supported by the DoD, PCRP, CDMRP PC100700 award to IAS, GP, DGM, SS and AD.

**Press Releases**

None

**Funding Applied for Based on Work Supported by this Award**

None

**Employment or research opportunities applied for and/or received based on experience/training supported by this award**

This award supports the employment and post-doctoral training of Shashwat Sharad, PhD. He has contributed to the qRT-PCR analysis of *PMEPA1*, *PSA* and *GAPDH* genes in 77 prostate.

The Research Assistant position of this award, in part supports the salary of Ms. Wei Huang, MS., a full-time employee of CPDR. She has experience in key techniques pertinent to this proposal. Ms. Huang continues the quality control of qRT-PCR reagents and assists in the completion of all qRT-PCR experiments. Also, she has completed the DNA sequence confirmation of PCR ARP amplicons.

Biostatistician Yongmei Chen, MD, MPH. (5%) effort performs the analysis of clinic-pathology data correlations. Correlation analyses include the qRT-PCR and IHC data towards developing the ARP cumulative index.

**List of Personnel (not salaries) receiving pay from the research effort**

PI: Albert Dobi, PhD.

Co-I: Gyorgy Petrovics, PhD.

Biostatistician: Yongmei Chen, MD, MPH.

Post-doctoral Fellow: Shashwat Sharad, PhD.

Research Assistant: Wei Huang, MS.

## **CONCLUSIONS**

The central hypothesis of the proposal that AR function in prostate tumor cells can be defined by measuring the expression of a panel of AR regulated genes. Towards defining the expression levels of AR regulated genes in prostate cancer we have completed the evaluation of mRNA expression in 77 patients by monitoring *ERG*, *PSA*, *PMEPA1* and *GAPDH* levels.

Evaluation of the ARP protein panel (*ERG*, *NKX3.1* and *PSA*) in 40 whole-mounted prostate specimens indicated remarkable accuracy in identifying BRC and metastasis in tumors with PD morphology (14 out of 15 BRC/metastasis). In contrast the ARP gene expression panel performs better in predicting favorable outcome in tumors with WD morphology, precisely identifying 13 cases with no BRC and no metastasis out of 14 cases with intact AR function.

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